Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol. 7, No. 14, 2017
www.iiste.org

Molecular Detection and Identification of Aspergillus fumigates from Goats in Waist Governorate of Iraq

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Abstract
A total of 100 samples of goats include 50 samples were collected from each tracheal swabs, and vaginal swabs from slaughter house and herds of goats in Waist province. During these studied, molds were isolated and identification depending on the cultural characteristic, microscopic feature and conventional Polymerase chain reaction (PCR). This study showed increase in the isolation of oppurtusinstic molds with increase age of animals, which observation from this study on goats found highest percentage of molds in those with ages between 3-5 years, while decrease percentage in animals that ages less than six months. The results showed significant differences (P<0.01) in molds isolation with increases animals ages in goats. Through study found *Aspergillus* spp. were predominant species. The most isolate of opportunistic molds were from tracheal swabs at percentage 63.6%, were significantly different (P<0.01) than vaginal swabs. The results of molds isolation showed the *A.fumigatus* and *A.niger*, were more isolated at percentage 40.9% and 22.8% respectively, followed by *A.terrus* (18.2%), *A.flavus* (9.1%), *Aspergillus* spp (4.5%) respectively, also isolated *Penicillium* spp. at percentage (4.5%). Showed result Significant differences (P<0.01) were found in isolation of molds between tracheal swabs, and vaginal swabs. The results of the PCR amplification of the rRNA gene showed that, this gene was present in 9 samples out 22 positive samples which isolation from the goats with a PCR product size of approximated 380 bp, while 13 samples out 22 positive samples showed negative results for the presence of this gene as indicated by the absence of the PCR products. The conclusion of present investigation indicated *Aspergillus fumigates* the most dominant molds isolated from tracheal swabs in goats, and this may be indicating a high contamination and exposure to *Asperigllus* spores. Molecular methods have revealed the important diversity of *Aspergillus* species within these action *Fumigati*.
Keywords: *A.fumigatus*, Goats in Waist Governorate, Polymerase chain reaction (PCR), rRNA gene

1. Introduction

"Aspergillosis is a spectrum of diseases that may be caused by a number of *Aspergillus* species which are ubiquitous saprobes in nature, and aspergillosis are found worldwide. The genus *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man and animal. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* , *Aspergillus niger and A.terreus*". *(*Pattron, 2006 and Steinbach *et al.,* 2012).

Aspergillus causes diseases of respiratory system by the inhalation of *Aspergillus* conidia. The clinical manifestations of pulmonary aspergillosis are many ranging from harmless saprophytic colonization to a cute invasive disease (Zhao *et al*, 2010 and Seyedmousavi *et al*, 2015). In immunocompromised led to invasive aspergillosis symptoms including fever, cough, dyspnea and hemoptysis. Hyphae invade the lumens and walls of blood vessels causing thrombosis, infarction, and necrosis from the lung. The disease may spread to the gastrointestinal tract, kidney, liver, brain or other organs producing abscesses and necrotic lesion (Radostits *et al*., 2007).

Aspergillus spp. are rapidly growing mould with septate hyphae. Many have highly colored colonies ranging from bluish –green through yellow to black due to the profuse production of pigment spores conidia (Fraga *et al*, 2008). *Aspergillus* spp. can causes disease in several ways. They can be the invasive cause of mycotoxicoses and are involved in allergic reactions in human (Brooks *et al*., 2007).

Aspergillus fumigatus cause many diseases in domestic animals. In dog it causes a disease in mucous membranes, guttural pouchin in horses, lung and air sac in poultry, and mastitis and abortion in bovine (Tell, 2005). *Aspergillus* species can be identified by the characteristic sporing head seen on microscopy and the strong color of the spores which is conferred on the colony. For example, *A.fumigatus* produces a deep blue-green colony, *A.flavus* light to yellow green, *A. terreus* cinnamon to beige or sandy brown, and *A. niger* dark brown to black .Various morphological features associated with the sporing head allow accurate identification (Ryan and Ray, 2004**).** Therefore the aim of present study to Molecular detection and identification of *Aspergillus fumigates* from goats.

2. Material and Methods

2.1. Samples Collection

A total of 100 samples in a ratio of 50 samples for each of tracheal, and vaginal swabs were collected from 100

goats, from slaughter house and herds of goats in Waist province.

2.2. Tracheal Swabs

Fifty samples of tracheal swabs have been collected from goats. The tracheal swabs were collected by sterile cotton swab after slaughter of animal and empted of viscera, then made incision in the lower part of tracheal and inter the sterile cotton swab in the lumen of tracheal. These swabs were transferred to the laboratory for diagnosis after adding few drops of sterile distilled water. In the laboratory the swabs were directly inoculated on to plates of Sabouraud dextrose agar with chlormphenicol, and incubated duplicated culture at 30 °C for 2 weeks (Koneman and Roberts, 1985)

2.3. Vaginal Swabs

Fifty samples were taken from female animals (50 goats) by sterile cotton swab from vagina, and then transferred to the laboratory for diagnosis after adding few drops of sterile distilled water in the laboratory.The swab was directly inoculated onto plates of SDA with chlormphenicol, and duplicated cultures were incubated at 30 °C cultures were maintained for 2 week (Koneman and Roberts, 1985).

2.4.Morphology Diagnosis

The identification was done depended on the shape and color of the fungus on the plate, and examined under the microscope. For appearance of the fungus, small portion from the fungal growth was taken, mixed with one drop of lacto phenol cotton blue and covered with cover slip then examined under (40X) by the microscope (Chandler *et al*., 1980;Ellis, 1994).

2.5. DNA isolation from culture

Fungal strains were grown on Sabouraud agar at 28°C at least 5 days to produce a visible colony, and a tiny portion of the colony was transferred directly to the PCR tube, followed by the steps installed and attached with the QIAamp DNA mini kit (Qiagen, Germany) was used to complete the extraction, following the manufacturer's protocol from the step. DNA yield was stored at (-20° C) until use.

2.6. Determination of genomic DNA concentration and purity

The concentration and purity of the purified DNA were quantified by nanodrop instrument, by following the instruction of the manufacturer(Act Gene NAS99) Briefly, 3µl was aspirated using special tips (Aeroject tips 10µl) and inserted in specified socket in the machine, DNA was quantified by the refractive index using the wave length 260nm, 280nm. DNA concentration was calculated with the OD260nm . The purity was estimated with the OD260nm/OD280nm ratio, a ratio of ~1.8 was generally accepted as "pure" DNA, indicating a low degree of protein contamination.

2.7. Gel electrophoresis

DNA samples were electrophoresed by horizontal agarose gel electrophoresis according to (Sambrook *et al*., 2001) as follows: Agarose(Promega, USA) at a concentrations of 2% was prepared, the agarose solution was left to cool at 55°C, then(0.5µl) of ethidium bromide solution(Promega, USA) was added, Agarose solution poured into the taped plate. A comb was placed near one edge of the gel. The gel was left to harden until it became opaque; each of the comb and tape were removed gently. TBE buffer (1X) prepared was poured into the gel tank and the slab was placed horizontally in electrophoresis tank. About 3 microliters of loading buffer prepared was applied to each 7 µl of DNA sample wells were filled with the mixture by a micropipette, PCR products were directly applied. Power supply was set at (5 V/cm (70) for 1 hr) for genomic DNA and PCR products electrophoresis. When the electrophoresis was finished the gel was exposed to UV light using UV transilluminator and then photographed using digital camera.

2.8. Primer design.

The specific of oligonucleotide primer sequences "were designed based on the sequence data for the internal transcribed spacer (ITS) region of the rRNA gene .Species-specific primer pairs— AFUM1-AFUM2 The forward primers (primer 1 of each pair) were designed within the ITS1 region, and the reverse primers (primer 2) were designed from the ITS2 region". This primer sequences were taken from (Guizhen and Thomas, 2002). and synthesized in Alpha DNA® (Canada) were used in conventional PCR to detect the *Aspergillus fumigates* as shown in. **(Table:1)**

Table (1): Primers sequences with their relevant product size

Gene target	Primer name		Primer sequence (5' ⟶ 3')	GenBank Accession No	Product Size(bp)
(ITS) region of the rRNA gene	AFUM1	F	CGC CGA AGA CCC CAA CAT GAA CGC	AF176662, AF078889	385
	AFUM2	R	TAA AGT TGG GTG TCG GCT GGC		

2.9. Polymerase chain reaction PCR

The specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of (ITS) region of the rRNA gene to detect the *Aspergillus fumigates*, the primers (AFUM1 and AFUM2) were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within each single pre-mixed PCR reaction tube, 2µl from each forward primer and reverse primer were added. Five microliter of DNA template was added for each reaction tube. Twelve and a half microliters of GoTaq® Green Master Mix(Promega, USA) was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease –Free as shown in table no.2, tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. PCR mixture without DNA template(non-template negative control) were used as negative control. The tubes were placed on the PCR machine and the PCR program, with the right cycling conditions pre-installed, was started. Cleaver Scientific Thermal Cycler TC32/80 was used for all PCR amplification reactions. The PCR thermocycler program used with The PCR thermocycler program used with (ITS) region of the rRNA gene was designed on the basis of published paper as shown in table no. 3.

Table (2): Composition of PCR reaction mixture used for amplification of (ITS) region of the rRNA gene

	Components	Volume /µl	Final concentration
(ITS) region	Green Master Mix 2x	12.5 µl	1x
	Forward primer, 10µM	2 µl	0.2 µM
	Reverse primer, 10µM	2 µl	0.2 µM
	DNA template	5 µl	
	(DNAse free) water	3.5 µl	
	Total volume	25 µl	

Table (3): The PCR thermo cycler program for (ITS) region of the rRNA gene

Steps	Temperature	Time	No. Cycles
Initial denaturation	94°C	5 min	
Denaturation	94°c	20 sec	
Annealing	56°c	20 sec	40
Extension	72°c	20 sec	
Final extension	72°c	5 min	
Hold	4°c		

2.10. PCR Gel Electrophoresis

Electrophoresis was done as stated earlier**.** Five microliters of the 100bp DNA ladder(Promega, USA) were mixed with one microliter of blue/orange 6X loading dye(Promega, USA) and subjected to electrophoresis in a single lane. Served as marker during PCR products electrophoresis. The gel was exposed to UV using UV light transilluminator and then photographed using digital camera (Sony-Japan).

2.11. Statistical Analysis

"In order to determine the statistical significances among different variables SPSS program (Statistical program for social sciences)" version 11, was used. Chi-square and RLSD by ANOVA test (One-Way Analysis of Variance) were applied.

3. RESULTS AND DISCUSSION

3.1 Age of Goats

This study showed increases of molds isolation in goats with the increase age of animals. Highest percentages were in age between 3-5 years. While less percentage were less than six months (Table 4). Statistical analysis showed significant differences (P<0.01) in molds isolation with increases in animals ages. In relation of occurrence of opportunistic mold with age, the present study revealed increase of mold isolation from animals with the increase of animals' age. The highest percentage was with age more than 6 years compares with one year with significant differences (P<0.01). These results agree with Al-Maadidhi (2008) who studied the fungal type infection in reproductive system in ewes, he found that the percentage of infection raise with increase in the age animal. Other studies showed that the percentage of infection increase of with increase in animals age. Wiserman *et al*., (1984) they found that the percentage of systemic mycotic infection increased with increasing of animal ages. Samaka (2000) also found that the animals that ages more than 6 years the percentage of infection was 48.8 % whereas less percentage in animals that ages lower than 5 years. The causes may be due to the animals in this ages have the increase chance to environment contact and also the animals are sexually active in this age which may be contamination through coating, parturition and abortion with other microorganism .

Table (4): Number positive samples in relation with age in goats

Age / samples	Less than 6 months	1 year	2 years	3 years	4 years	5 years
Tracheal	0	1	2	2	4	5
Vaginal	0	1	1	1	2	3
Total positive	0	2	3	3	6	8
% positive	%0	%9.1d	%13.6cd	%13.6cd	%27.3bc	%36.4 ab

RLSD=P< 0.01

3.2. Morphology Diagnosis

The results of molds isolation from goats showed the total positive samples in goats 22(22%) out of 100 samples. Also through this study the most isolate of opportunistic molds were from tracheal swabs 14(63.6%) out of 22 positive samples, the tracheal swabs were significant differences (P<0.01) than vaginal Swabs**.** The most common isolates were *Asperigellius spp*. particular *A.fumigatus* at percentage (40.9 %) followed by *A.niger* 22.8%, *A.terrus* 18.2%, *A.flavus* 9.1%, *Aspergillus* spp. and *Penicillium* spp. 4.5% respectively (Table 5 and Figures 1). These results agreed with Samaka (2000), who notes *Asergillus* spp. isolates more frequently from tracheal swabs in cattle and sheep, in cattle he found *A.fumigatus* more frequent in percentage 42% followed by *A.niger* 26.3% , while in sheep also found *A.fumgatus* more frequent in percentage of 33.3% . Other studies found that *Aspergillus fumigatus* more frequent than other molds, when mastitis milk of ewe was examined (Al-kubaysi, 2000). Whereas Ali and Khan (2006) observated the mycotic abortion is an important reproduction problem in cattle and buffaloes.

They found that the chief fungus associated with mycotic abortion was *Aspergillus fumigatus* which has been recorded from over 60% of cases, also notes no clinical symptoms have been observed in the dam either before or after abortion, but clinical diagnosis can be made on the pathological appearance of placenta and particularly the cotyledons and also on the presence of foetal skin lesions. Through our result the highest percentage of isolation of opportunistic systemic fungi showed in goats were from tracheal at percentage 63.6%. The tracheal were significant differences (P<0.01) than vaginal swabs.

The tracheal swabs as a part of respiratory system may be this system which contact directly with external system environment led to easily entry of the spores to the respiratory system by inhalation and available environment stander from temperature and moisture make from this system more exposure to the mycotic infection (Brook *et al*., 2007).

Table (5): Number and percentage of molds isolates in goats samples.

Samples / Species	Tracheal swabs	Vaginal swabs	Total positive	Total % positive
A.fumigatus	6	3	9a	40.9%
A.niger	3	2	5ab	22.8%
A.terreus	2	2	4ab	18.2%
A.flavus	1	1	2ab	9.1%
Aspergillus. spp	1	0	1b	4.5%
*Pencillium.*spp	1	0	1b	4.5%
Total isolation	14a	8ab	22	100%
Total % positive	63.6%	36.4%		

↓ RLSD=P< 0.01
→ RLSD=P< 0.01

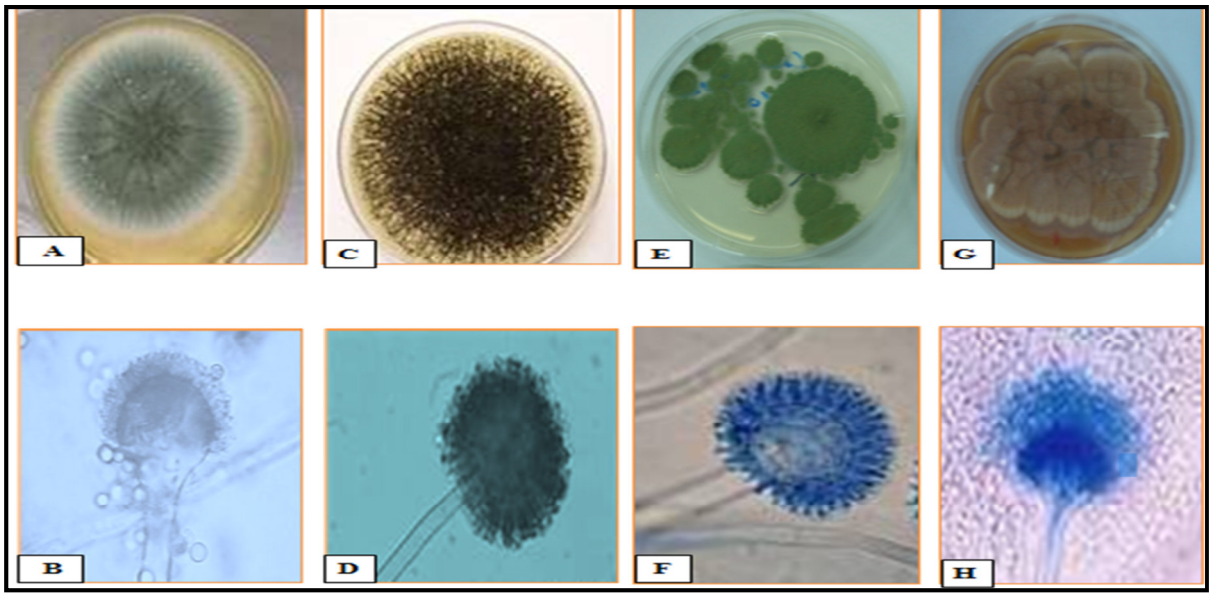
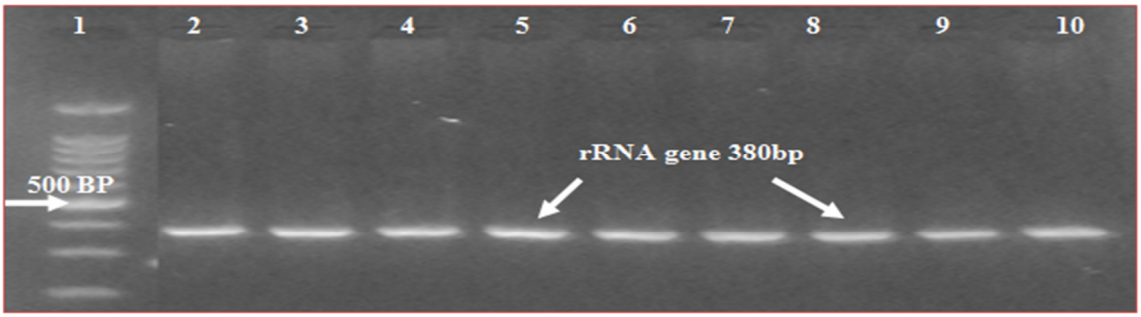


Figure (1): Shows the morphological and microscopic characteristics of some important types of *Aspergillus* species 1-*A.fumigatus* (macroscopic A & microscopic B). 2-*A.niger* (macroscopic C & microscopic D). 3- *A.flavus* (macroscopic E & microscopic F). 4- *A.terreus niger* (macroscopic G & microscopic H).

3.3. Molecular diagnosis

The specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of (ITS) region of the rRNA gene for *Aspergillus fumigates*. The results of the PCR amplification of the rRNA gene showed that, this gene was present in 9 samples out 22 positive samples which isolation from the goats with a PCR product size of approximated 380 bp, while 13 samples out 22 positive samples showed negative results for the presence of this gene as indicated by the absence of the PCR products in their relevant lanes. (Figure. 2).

For many purposes, this rapid method has become routine for the amplification of DNA from cultures of *Aspergillus* species." The molds were much less amenable to direct amplification, perhaps because of more intractable cell walls, abundant endogenous nucleases, inhibitors of the PCR, or other factors. There was a tendency for younger mold cultures to be more PCR positive than older cultures".



Figure(2): Gel electrophoresis(2% agarose,7v/cm2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane(2-10): Positive sample for *Aspergillus fumigates* (rRNA gene 380bp).

4. Conclusion

conclusion of present investigation indicated *Aspergillus fumigates* the most dominant molds isolated from tracheal swabs in goats, and this may be indicating a high contamination and exposure to *Asperigllus* spores. Molecular methods have revealed the important diversity of *Aspergillus* species within these action *Fumigati*.

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